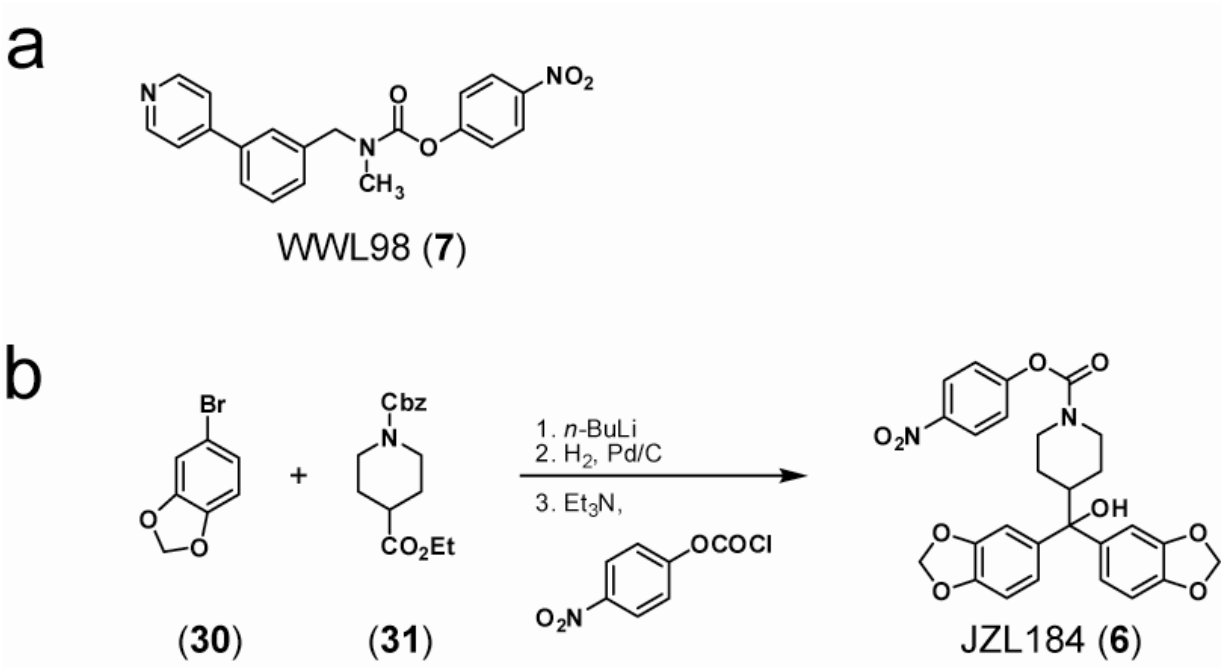
SELECTIVE BLOCKADE OF 2-ARACHIDONOYLGLYCEROL HYDROLYSIS PRODUCES CANNABINOID BEHAVIORAL EFFECTS

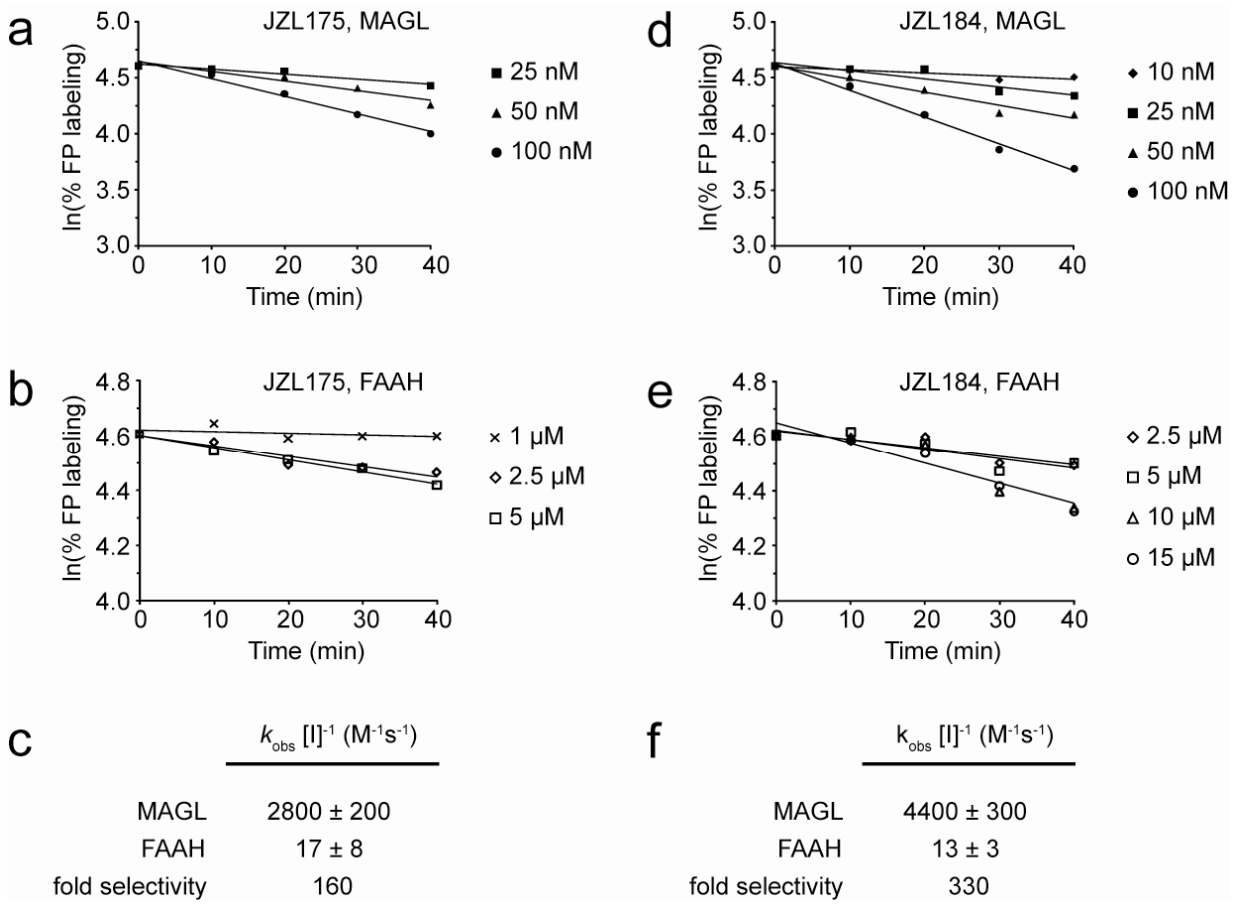
Jonathan Z. Long, Weiwei Li, Lamont Booker, James J. Burston, Steven G. Kinsey, Joel E. Schlosburg, Franciso J. Pavón, Antonia M. Serrano, Dana E. Selley, Loren H. Parsons, Aron H. Lichtman, Benjamin F. Cravatt

SUPPLEMENTARY INFORMATION

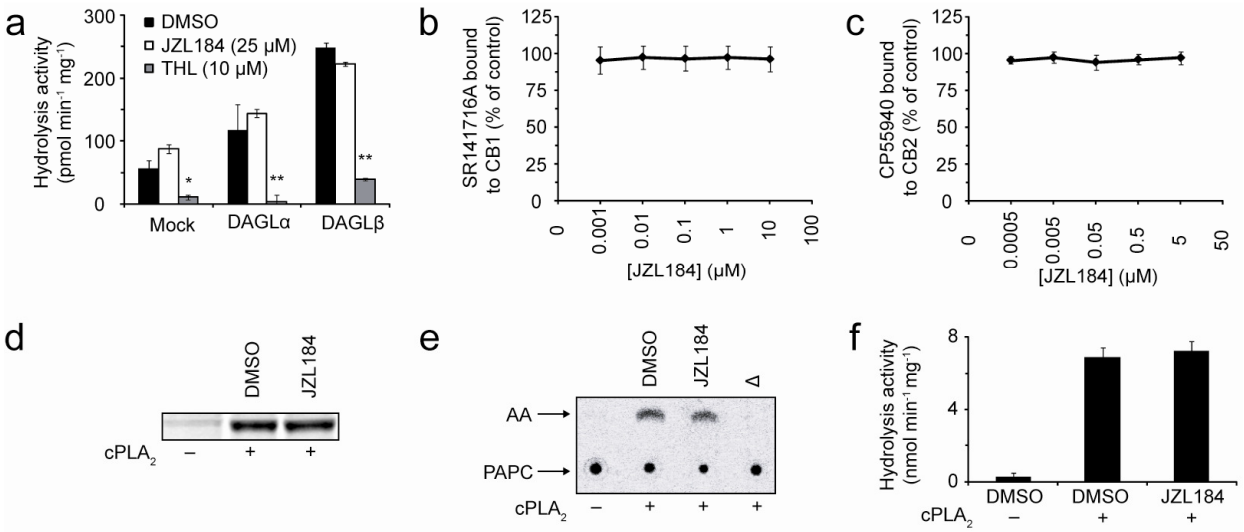
Contents:



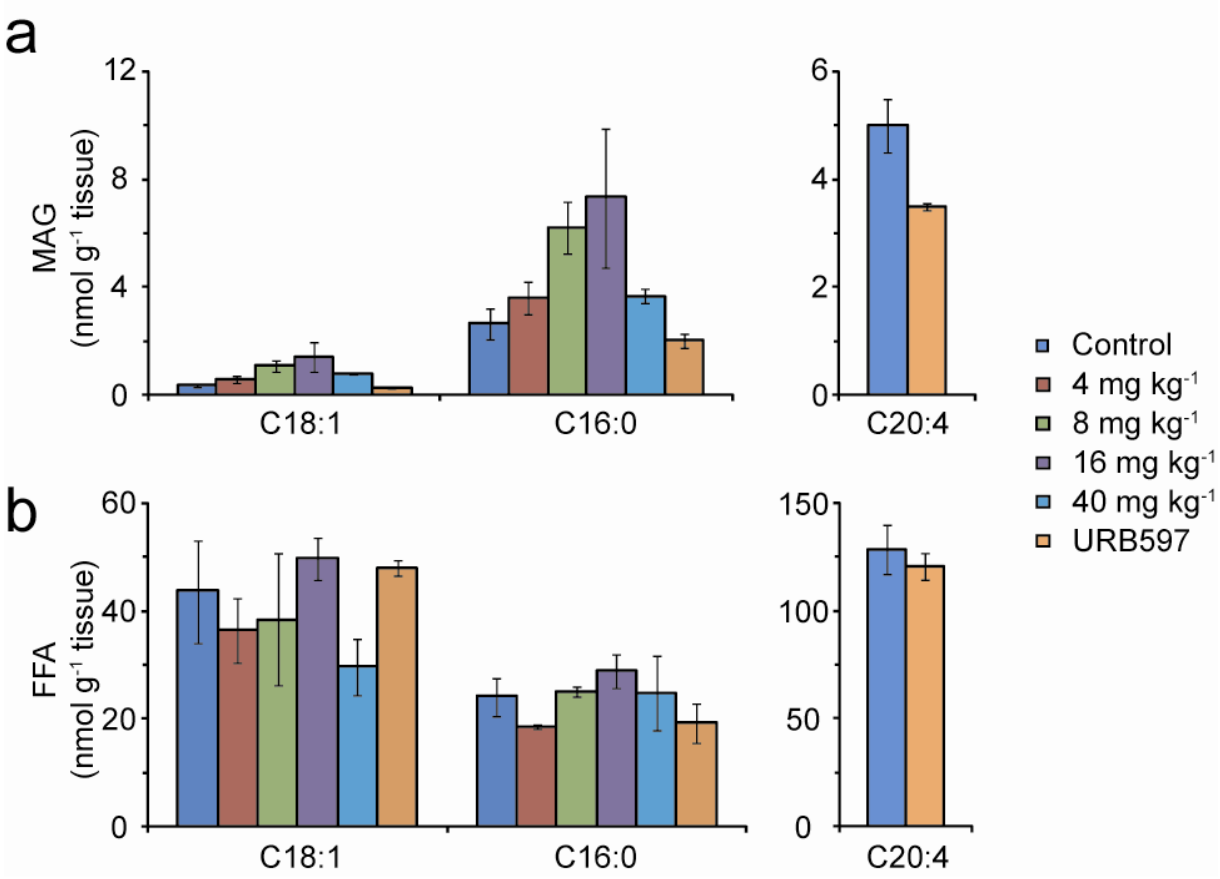
Supplementary Fig. 1. a, Structure of WWL98. **b**, Synthetic route for JZL184. Other carbamate inhibitors were synthesized by a similar route.



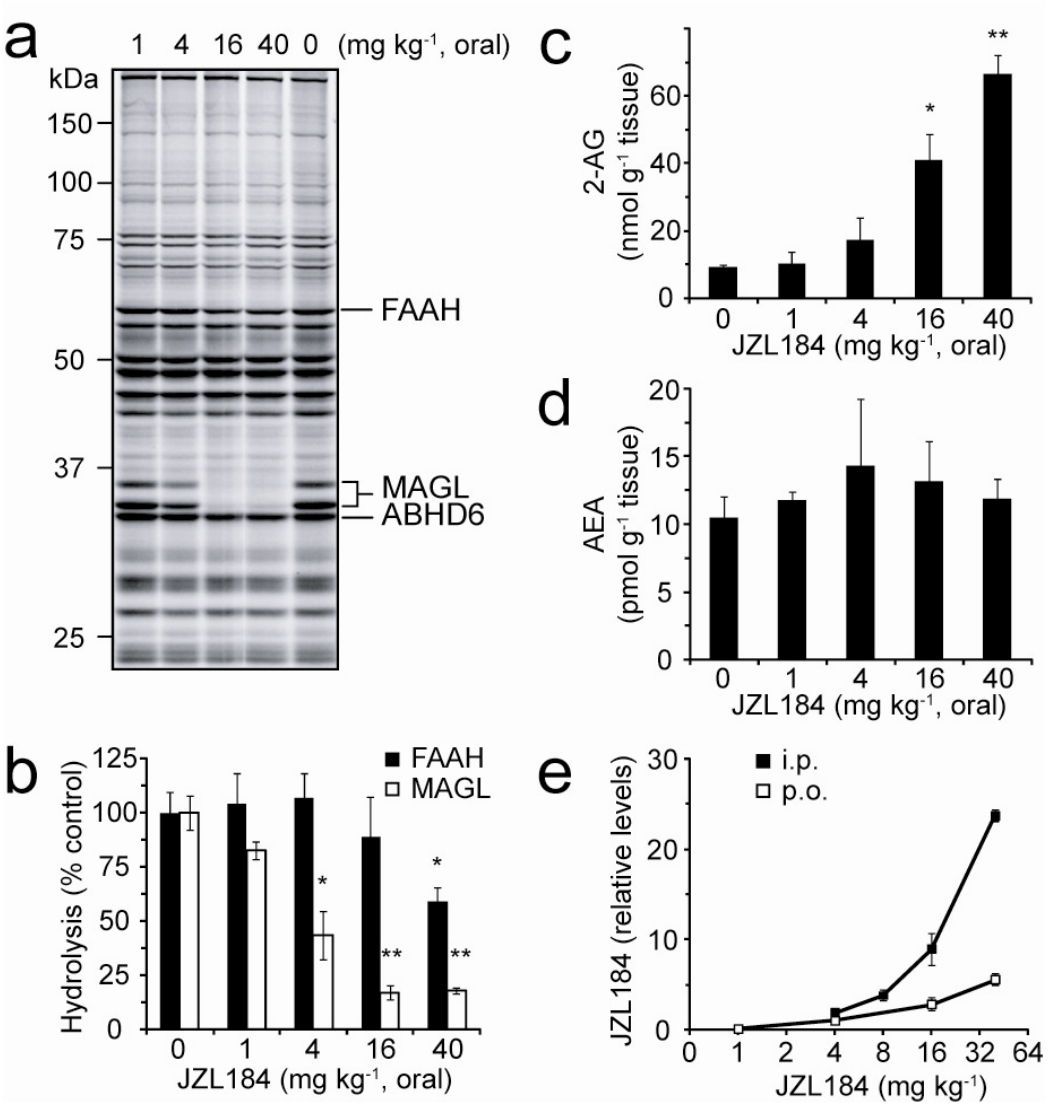
Supplementary Fig. 2. Time-dependent inhibition of MAGL and FAAH by JZL175 and JZL184. **a**, JZL175 inhibition of FP-rhodamine labeling of MAGL activity in mouse brain membrane proteome. **b**, JZL175 inhibition of FP-rhodamine labeling of MAGL activity in mouse brain membrane proteome. **c**, k_{obs} $[I]^{-1}$ values calculated for inhibition of MAGL and FAAH by JZL184. **d**, JZL175 inhibition of FP-rhodamine labeling of MAGL activity in mouse brain membrane proteome. **e**, JZL184 inhibition of FP-rhodamine labeling of MAGL activity in mouse brain membrane proteome. **f**, k_{obs} $[I]^{-1}$ values calculated for inhibition of MAGL and FAAH by JZL184.



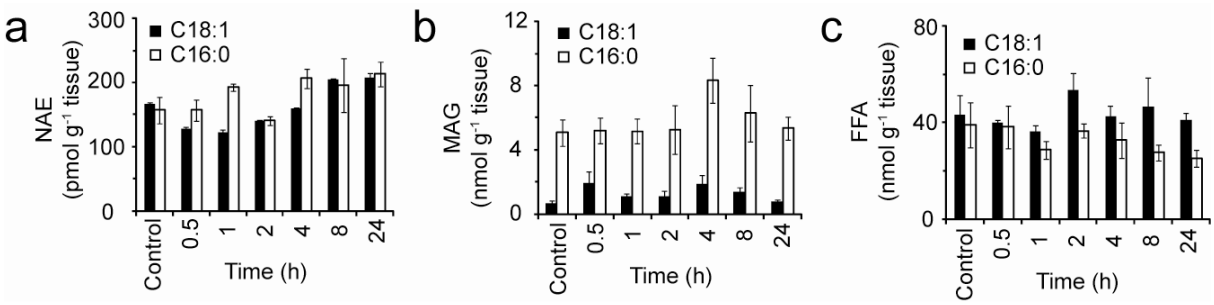
Supplementary Fig. 3. JZL184 does not interact with other components of the endocannabinoid system or other arachidonate-metabolizing enzymes. **a**, JZL184 (25 μM) did not inhibit the hydrolysis of 1-stearoyl-2-arachidonoyl-glycerol to 2-AG by the 2-AG biosynthetic enzymes DAGLα or DAGLβ when recombinantly expressed in HEK293 cells. The known DAGL inhibitor tetrahydrolipstatin (THL, 10 μM) was used as a positive control. **b** and **c**, JZL184 did not displace binding of ^{3}H-rimonabant from CB1 (**b**) or CB2 (**c**) receptors. **d** and **e**, JZL184 (20 μM) did not inhibit the activity of $cPLA_2$ when recombinantly expressed in HEK293 cells as judged by FP-rhodamine labeling (**d**) or by a radiolabeled TLC assay (**e**) that monitored the conversion of [^{14}C-arachdonoyl] 1-stearoyl-2-arachidonoyl-sn-glycerol-3-phosphocholine (PAPC) to arachidonic acid (AA). Mock transfected cells ($cPLA_2$, –) or heat denatured transfected cells ($cPLA_2$ +, heat) showed negligible amounts of hydrolysis activity. **f**, quantification of the hydrolysis activity from the TLC assay. Data are presented as means ± SEM of three independent experiments.



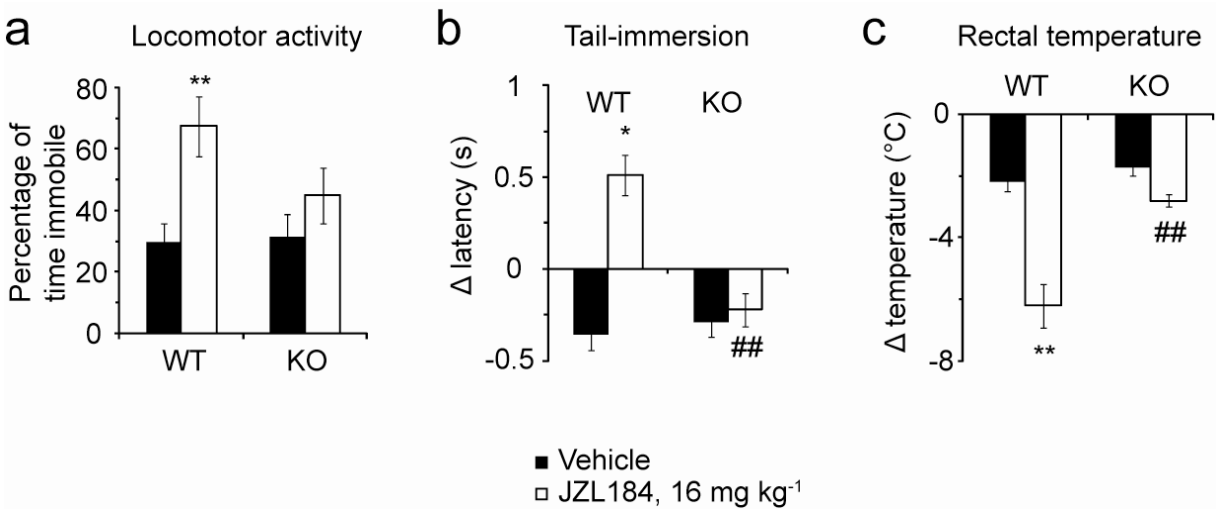
Supplementary Fig. 4. Brain lipid measurements for dose-response study of JZL184. No significant changes were observed in brain levels of C18:1 or C16:0 MAGs (**a**) or free fatty acids (**b**) was observed at 4 hrs following administration of JZL184 (4-40 mg kg^{-1}, i.p.).



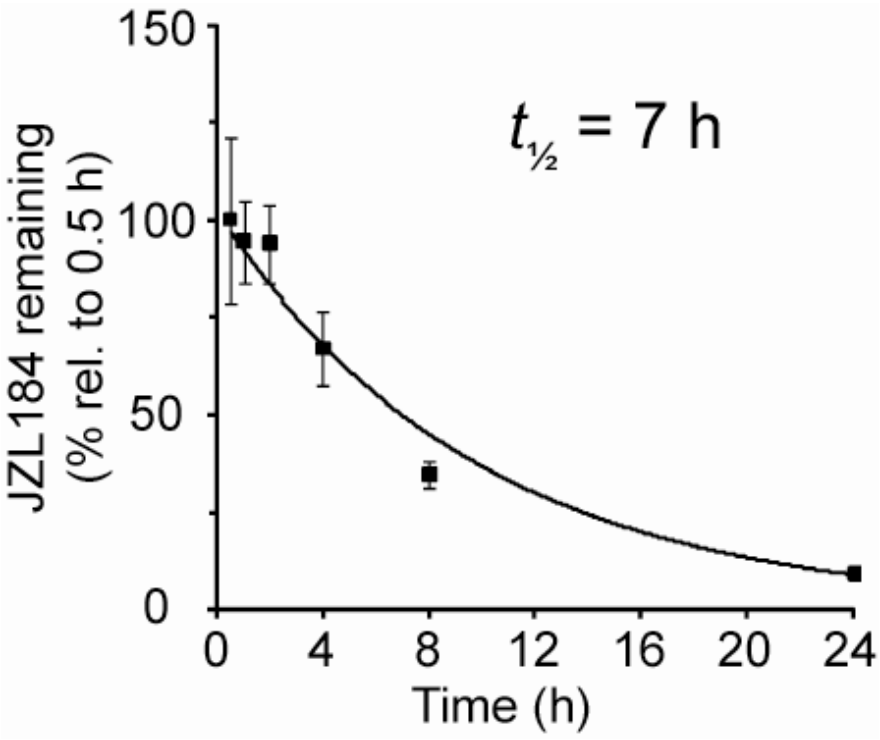
Supplementary Fig. 5. Oral administration of JZL184. **a** and **b**, Serine hydrolase activity profiles (**a**) and MAGL and FAAH activities (**b**) of brain membranes prepared from mice treated with JZL184 at the indicated doses (1-40 mg kg^{-1}, p.o.) for 4 h. **c** and **d**, Brain levels of 2-AG (**c**) and AEA (**d**) from mice treated with JZL184 (1-40 mg kg^{-1}, p.o.) for 4 hrs. **e**, Relative brain levels of JZL184 from mice treated either by i.p. or p.o. administration. For **b**-**d**, *, $p < 0.05$; **, $p < 0.01$ for inhibitor-treated versus vehicle-treated animals. Data are presented as means ± SEM. n = 3-5 mice per group.



Supplementary Fig. 6. Time course of brain lipid measurements following JZL184 treatment in mice. No significant changes were observed in brain levels of C18:1 or C16:0 NAEs (**a**), MAGs (**b**), or free fatty acids (**c**) throughout a 24 h time course following administration of JZL184 (16 mg kg^{-1}, i.p.).



Supplementary Fig. 7. Comparison of behavioral effects of JZL184 in $CB1^{+/+}$ and $CB1^{-/-}$ mice. JZL184 (16 mg kg^{-1}, i.p.) produced hypomotility (**a**), hypoalgesia (**b**), and hypothermia (**c**) in $CB1^{+/+}$ mice, but not $CB1^{-/-}$ mice. No catalepsy was observed in any of the mice. Pre-injection tail withdrawal latencies (mean ± SEM) in $CB1^{+/+}$ and $CB1^{-/-}$ mice were 1.26 ± 0.08 and 1.24 ± 0.03 s, respectively. Pre-injection rectal temperatures (mean ± SEM) in $CB1^{+/+}$ and $CB1^{-/-}$ mice were 37.9 ± 0.1 and 37.6 ± 0.1°C, respectively. *, $p < 0.05$; **, $p < 0.01$ for vehicle versus JZL184-treated $CB1^{+/+}$ mice. #, $p < 0.05$; ##, $p < 0.01$ for JZL184-treated $CB1^{-/-}$ versus JZL184-treated $CB1^{+/+}$ mice. Data are presented as means ± SEM. $n = 8$ mice/group.



Supplementary Fig. 8. Time course analysis of JZL184 levels in mouse brain. JZL184 was administered at 16 mg kg^{-1}, i.p. From these data, an estimated half-life of 7 h was calculated.

ENSEMBL Identifier	Common Name	Abbreviation	VEH	JZL184	*p*-value
ENSMUSG00000027698	Arylacetamide deacetylase-like 1	AADACL1	121.3 ± 16.1	121.6 ± 20.4	0.990
ENSMUSG00000025153	Fatty acid synthase	FAS	108 ± 14.8	120.3 ± 8.1	0.507
ENSMUSG00000032046	Alpha/beta hydrolase domain containing 12	ABHD12	94.3 ± 7.6	94.3 ± 21.8	1.000
ENSMUSG00000033174	**Monoacylglycerol lipase**	**MAGL**	**59.6 ± 12.8**	**12.3 ± 1.2**	**0.021**
ENSMUSG00000025277	Alpha/beta hydrolase domain containing 6	ABHD6	52.3 ± 8.3	30.3 ± 1.8	0.062
ENSMUSG00000024127	Prolyl endopeptidase-like	PREPL	36.6 ± 2.9	28.6 ± 3.1	0.139
ENSMUSG00000034171	**Fatty acid amide hydrolase**	**FAAH**	**30 ± 4.7**	**12.3 ± 3.2**	**0.037**
ENSMUSG00000019849	Prolyl endopeptidase	PREP	23.3 ± 2.3	26.3 ± 3.8	0.541
ENSMUSG00000002475	Alpha/beta hydrolase domain containing 3	ABHD3	17.3 ± 2.9	23 ± 4.0	0.319
ENSMUSG00000070889	Glycerophosphoinositol deacylase	GPID	22 ± 3.5	22.3 ± 0.3	0.929
ENSMUSG00000007036	HLA-B associated transcript 5	BAT5	21.6 ± 2.6	15.6 ± 2.1	0.157
ENSMUSG00000041763	Tripeptidylpeptidase 2	TPP2	16 ± 1.0	14.3 ± 1.4	0.398
ENSMUSG00000036257	Patain-like phospholipase domain containing 8	PNPLA8	15.3 ± 4.6	15.6 ± 2.3	0.952
ENSMUSG00000023328	Acetylcholinesterase	AChE	15.3 ± 1.6	13 ± 1.0	0.296
ENSMUSG00000023913	Phospholipase A2 group 7	PLA2G7	15 ± 2.5	11.6 ± 0.6	0.270
ENSMUSG00000021226	Acyl-CoA thioesterase 2	ACOT2	14.3 ± 2.6	12.3 ± 0.8	0.507
ENSMUSG00000030718	Protein phosphatase methylesterase 1	PPME1	13.6 ± 1.7	13.6 ± 2.3	1.000
ENSMUSG00000017760	Cathepsin A	CTHA	10 ± 1.5	13.3 ± 2.0	0.259
ENSMUSG00000033157	Alpha/beta hydrolase domain containing 10	ABHD10	13.3 ± 2.3	9 ± 2.0	0.238
ENSMUSG00000032393	Dipeptidylpeptidase 8	DPP8	10.6 ± 2.0	12.3 ± 2.1	0.606
ENSMUSG00000004565	Neuropathy target esterase	NTE	8 ± 1.7	11.6 ± 0.3	0.106
ENSMUSG00000069922	Carboxylesterase 31	CES31	1.6 ± 1.6	10 ± 4.3	0.149
ENSMUSG00000072949	Acyl-CoA thioesterase 1	ACOT1	9 ± 1.1	10 ± 0.5	0.482
ENSMUSG00000001229	Dipeptidylpeptidase 9	DPP9	7.6 ± 2.3	9.3 ± 1.7	0.599
ENSMUSG00000003346	FAM108A1	FAM108A1	9.3 ± 0.8	7.6 ± 2.3	0.541
ENSMUSG00000047368	CGI67	CGI67	9 ± 1.5	7 ± 1.5	0.407
ENSMUSG00000039246	Lysophospholipase-like 1	LPL1	8.3 ± 3.7	8 ± 2.0	0.942
ENSMUSG00000027428	Retinoblastoma binding protein 9	RBBP9	7.6 ± 0.3	8 ± 0.0	0.374
ENSMUSG00000032590	Acylaminoacid releasing enzyme	AARE	7.3 ± 1.3	4.6 ± 0.6	0.148
ENSMUSG00000061119	Prolylcarboxypeptidase	PRCP	7.3 ± 1.2	4.6 ± 1.2	0.192
ENSMUSG00000028670	Acyl protein thioesterase 2	APT2	6.6 ± 1.2	5 ± 0.0	0.238
ENSMUSG00000031903	1-O-acylceramide synthase	LYPLA3	6.3 ± 0.8	6.6 ± 0.6	0.778
ENSMUSG00000040532	Alpha/beta hydrolase domain containing 11	ABHD11	6.3 ± 0.3	5.6 ± 1.2	0.621
ENSMUSG00000036833	Neuropathy target esterase-like	NTE-like	6 ± 1.0	6 ± 1.1	1.000
ENSMUSG00000038459	2210412D01	2210412D01	5.3 ± 1.3	6 ± 1.7	0.776
ENSMUSG00000005447	Platelet activating factor acetylhydrolase 1B gamma	PAFAH-1Bg	5.6 ± 1.3	3.6 ± 2.3	0.498
ENSMUSG00000003131	Platelet activating factor acetylhydrolase 1B beta	PAFAH-1Bb	2.6 ± 1.3	5 ± 1.0	0.234

Supplementary Table 1. Full names and Ensembl identifier codes for brain membrane serine hydrolase enzymes identified in ABPP-MudPIT experiments (as shown in **Fig. 3c**).

SUPPLEMENTARY METHODS

General synthetic methods. All reagents were purchased from Sigma-Aldrich, Acros, Fisher, Fluka, or Maybridge and used without further purification, except where noted. Dry solvents were obtained by passing commercially available pre-dried, oxygen-free formulations through activated alumina columns. All reactions were carried out under a nitrogen atmosphere using oven-dried glassware unless otherwise noted. Flash chromatography was performed using 230-400 mesh silica gel. NMR spectra were recorded in $CDCl_3$ on a Varian Inova-400 or a Bruker DMX-600 spectrometer and were referenced to trimethylsilane (TMS) or the residual solvent peak. Chemical shifts are reported in ppm relative to TMS and *J* values are reported in Hz. High resolution mass spectrometry (HRMS) experiments were performed at The Scripps Research Institute Mass Spectrometry Core on an Agilent mass spectrometer using electrospray ionization-time of flight (ESI-TOF).

Synthesis of JZL184. To a stirring solution of 4-bromo-1,2-methylenedioxybenzene (**6**, 2.01 g, 10 mmol) in anhydrous THF (30 ml) was added *n*-BuLi (3.8 ml, 2.6 M in toluene, 9.9 mmol) dropwise at -78°C. After stirring 1.5 hrs at the same temperature, a solution of ethyl *N*-Cbz-isonipecotate (**7**, 1.02 g, 3.5 mmol) in anhydrous THF (10 ml) was added dropwise and an additional portion of THF (3 ml) was used to quantitate the transfer. After the reagent addition was complete, the cooling bath was removed and the suspension gradually turned into a clear solution. After 3 hrs, the reaction was quenched with water and diluted with EtOAc. The layers were separated and the organic layer was washed twice with water, once with brine, dried over Na_2SO_4, and concentrated *in vacuo*. Purification of the crude oil via flash chromatography (Hex:EtOAc = 6:1 then 4:1) gave the intermediate alcohol as a white solid (0.81 g, 47% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.29 (qd, *J* = 4, 12 Hz, 2H), 1.51 (bs, 2H), 2.38 (tt, *J* = 2.8, 12 Hz, 1H), 2.58 (s, 1H), 2.75 (t, *J* = 12 Hz, 2H), 4.19 (bs, 2H), 5.03 (d, *J* = 5 Hz, 2H), 5.85 (s, 4H), 6.70 (d, *J* = 8 Hz, 2H), 6.88-6.92 (m, 4H), 7.24-7.33 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 26.6, 44.4, 44.6, 67.1, 79.4, 101.1, 106.9, 107.9, 118.9, 127.9, 128.0, 128.5, 136.9, 140.1, 146.2, 147.7, 155.2; HRMS calculated for $C_{28}H_{27}NNaO_7$ $[M+Na]^+$ 512.1680, found 512.1670. A portion of this intermediate (0.72 g, 1.5 mmol) was dissolved in CH_2Cl_2/EtOH (1:1 v/v, 40 ml) and Pd/C (10%, 0.20 g) was added in one portion. The reaction was stirred under H_2 (1 atm)

overnight. After complete consumption of the starting material (12-24 hrs), the reaction was filtered and concentrated *in vacuo*. The resulting crude solid was dissolved in CH_2Cl_2 (30 mL) and to it was sequentially added Et_3N (2 ml, 15 mmol) and 4-nitrophenyl chloroformate (460 mg, 2.3 mmol). The reaction was stirred overnight. The next morning, the reaction was quenched with water and diluted with EtOAc. The layers were separated and the organic layer was washed thrice with 2N NaOH, once with brine, dried over Na_2SO_4, and concentrated *in vacuo*. Purification of the crude oil via flash chromatography (Hex:EtOAc = 6:1 then 4:1) gave JZL184 (**1**) as a pale yellow oil that turned into a yellow solid upon standing under vacuum (550 mg, 71% yield for two steps). The characterization of JZL184 is described in the main text.

Synthesis of JZL175. To a stirring solution of ethyl *N*-Cbz-isonipecotate (0.84 g, 2.9 mmol) in anhydrous THF (10 ml) was added 4-anisylemagnesium bromide (15 ml, 0.5 M in THF) dropwise at room temperature. After the reagent addition was complete, the reaction was heated to reflux overnight. The next morning, the reaction was quenched with water and diluted with EtOAc. The layers were separated and the organic layer was washed twice with water, once with brine, dried over Na_2SO_4, and concentrated *in vacuo*. Purification of the crude oil via flash chromatography (Hex:EtOAc = 6:1 then 2:1) gave the intermediate alcohol as a pale yellow solid (0.90 g, 69% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.24-1.33 (m, 2H), 1.54 (bs, 2H), 2.14-2.18 (m, 1H), 2.46 (t, J = 12 Hz, 1H), 2.77 (t, J = 11 Hz, 2H), 3.75 (s, 6H), 4.21 (bs, 2H), 5.06 (s, 2H), 6.81 (d, J = 9 Hz, 4H), 7.27-7.34 (m, 9H). This material was used without further characterization. A portion of this intermediate (55 mg, 0.18 mmol) was treated in a two step procedure with Pd/C and 4-nitrophenyl chloroformate in a manner analogous to the synthesis of JZL184. Purification of the crude oil via flash chromatography (Hex:EtOAc = 6:1 then 2:1) gave JZL175 as a pale yellow oil that turned into a yellow solid upon standing under vacuum (55 mg, 62% yield for two steps): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.40 (m, 2H), 1.65 (t, J = 11 Hz, 2H), 2.26 (bs, 1H), 2.55 (t, J = 12 Hz, 1H), 2.87 (t, J = 12 Hz, 1H), 3.00 (t, J = 13 Hz, 1H), 3.76 (s, 6H), 4.27 (t, J = 13 Hz, 2H), 6.84 (d, J = 9 Hz, 4H), 7.24 (d, J = 9 Hz, 2H), 7.34 (d, J = 9 Hz, 4H), 8.19 (d, J = 9 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 26.6, 26.9, 44.4, 45.2, 55.4, 79.2, 113.6, 113.7, 122.4, 125.1, 127.3, 137.8, 137.9, 144.8, 152.2, 156.5, 158.4; HRMS calculated for $C_{27}H_{27}N_2O_6$ $[M\text{-}H_2O+H]^+$ 475.1864, found 475.1864.

Synthesis of WWL152. To a stirring solution of *N*-Boc piperazine (100 mg, 0.54 mmol) in CH_2Cl_2 (5 ml) was sequentially added 4-nitrophenyl chloroformate (109 mg, 0.54 mmol) and Et_3N (75 µl, 0.54 mmol) at room temperature. After 3 hrs, the reaction was concentrated *in vacuo*. Purification of the crude mixture via flash chromatography (Hex:EtOAc = 3:1) gave the Boc-protected intermediate as a white solid (100 mg, 52% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.24 (d, *J* = 9 Hz, 2H), 7.31 (d, *J* = 9 Hz, 2H), 3.67 (bs, 2H), 3.54 (bs, 6H), 1.50 (s, 9H). To the intermediate (100 mg, 0.28 mmol) was added 1:1 v/v TFA:CH_2Cl_2 (2 ml) at room temperature. After 2 hrs, the reaction was concentrated *in vacuo*. The crude was used without further purification. To a stirring solution of 4-methoxy benzophenone (100 mg, 0.5 mmol) in EtOH (5 ml) was added $NaBH_4$ (38 mg, 1 mmol) at room temperature. The next morning, the reaction was poured onto water (10 ml), stirred for 1 hr, and then the product was filtered off and dried. To the crude alcohol in CH_2Cl_2 (5 ml) was added oxalyl chloride (40 µL, 0.46 mmol) dropwise at room temperature. After 2 hrs, the reaction was concentrated *in vacuo*. The crude was redissolved in CH_3CN (2 ml) and to it was sequentially added 4-nitrophenyl piperazine-1-carboxylate (58 mg, 0.23 mmol) and Et_3N (32 µl, 0.23 mmol). After the reagent addition was complete, the reaction was heated to reflux overnight. The next morning, the reaction was concentrated *in vacuo*. Purification of the crude mixture via flash chromatography (Hex:EtOAc = 1:1) gave WWL152 as a white solid (201 mg, 84% yield over 3 steps): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.24 (d, *J* = 9 Hz, 2H), 7.32 (d, *J* = 8 Hz, 4H), 7.27 (d, *J* = 9 Hz, 2H), 6.84 (d, *J* = 8 Hz, 4H), 4.22 (s, 1H), 3.76 (s, 6H), 3.66 (s, 2H), 3.57 (s, 2H), 2.45 (s, 4H); HRMS calculated for $C_{26}H_{27}N_3O_6$; ^{13}C NMR ($CDCl_3$, 150 MHz) δ 44.4, 44.9, 51.4, 51.6, 74.4, 115.7, 115.8, 122.4, 125.2, 129.3, 129.4, 137.60, 137.62, 145.0, 152.2, 156.3, 161.3, 162.9; HRMS calculated for $C_{24}H_{22}F_2N_3O_4$ $[M+H]^+$ 454.1573, found 454.1578.

Synthesis of WWL162. To 1-(bis(4-fluorophenyl)methyl)piperazine (29 mg, 0.1 mmol) in CH_2Cl_2 (2 mL) was sequentially added 4-nitrophenyl chloroformate (20 mg, 0.1 mmol) and Et_3N 14 µl, 0.1 mmol) at room temperature. After 3 hrs, the reaction was concentrated *in vacuo*. Purification of the crude mixture via flash chromatography (Hex:EtOAc = 3:1) gave WWL162 as a white solid (42 mg, 92% yield): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.24 (d, *J* = 9 Hz, 2H), 7.36 (m, 4H), 7.28 (d, *J* = 9 Hz, 2H), 7.00 (m, 4H), 4.29 (s, 1H), 3.68 (s, 2H), 3.59 (s, 2H), 2.44 (s, 4H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 44.5, 45.0, 51.4, 51.6, 55.3, 74.5, 113.9, 114.1, 122.4,

125.2, 127.8, 128.9, 134.5, 144.9, 152.3, 156.4, 158.7; HRMS calculated for $C_{26}H_{28}N_3O_6$ $[M+H]^+$ 478.1973, found 478.1977.

Preparation of mouse tissue proteomes. Mouse brains were Dounce-homogenized in PBS, pH 7.5, followed by a low-speed spin (1,400 x *g*, 5 min) to remove debris. The supernatant was then subjected to centrifugation (64,000 x *g*, 45 min) to provide the cytosolic fraction in the supernatant and the membrane fraction as a pellet. The pellet was washed and resuspended in PBS buffer by sonication. Total protein concentration in each fraction was determined using a protein assay kit (Bio-Rad). Samples were stored at -80°C until use.

Recombinant expression in COS7 or HEK293 cells. Briefly, full-length cDNAs encoding mouse serine hydrolases were purchased from OpenBioSystems (Huntsville, AL). cDNAs were either transfected directly (if available in a eukaryotic expression vector) or subcloned into pcDNA3 (Invitrogen). COS7 or HEK293 cells were grown to ~70% confluence in 10 cm dishes in complete medium (DMEM with L-glutamine, nonessential amino acids, sodium pyruvate, and FCS) at 37°C and 5% CO_2. The cells were transiently transfected by using the appropriate cDNA or empty vector control ("mock") and the FUGENE 6 (Roche Applied Science) transfection reagents following the manufacturers' protocols. After 48 hrs, the cells were washed twice with phosphate-buffered saline (PBS), collected by scraping, resuspended in 0.25 ml PBS, and lysed by sonication. The lysates were used in assays as whole-cell homogenates, except for DAGLα and DAGLβ, where the membrane fraction was used.

Enzyme activity assays. Briefly, 2-AG (100 µM) was incubated with mouse brain membrane (20 µg) or recombinant MAGL in COS7 cells (0.1 µg) in PBS (200 µl) at room temperature for 10 min. The reactions were quenched by the addition of 500 µL 2:1 v/v $CHCl_3$:MeOH, doped with 0.5 nmol PDA, vortexed, then centrifuged (1,400 x *g*, 3 min) to separate the phases. 30 µl of the resultant organic phase was injected onto an Agilent 1100 series LC-MSD SL instrument. LC separation was achieved with a Gemini reverse-phase C18 column (5 µm, 4.6 mm x 50 mm, Phenomonex) together with a pre-column (C18, 3.5 µm, 2 mm x 20 mm). Mobile phase A was composed of 95:5 v/v H_2O:MeOH, and mobile phase B was composed of 60:35:5 v/v/v *i*-PrOH:MeOH:H_2O. 0.1% ammonium hydroxide and 0.1% formic acid was included to assist in

ion formation in negative and positive ionization mode, respectively. The flow rate was 0.5 ml min^{-1} and the gradient consisted of 1.5 min 0% B, a linear increase to 100% B over 5 min, followed by an isocratic gradient of 100% B for 3.5 min before equilibrating for 2 min at 0% B. MS analysis was performed with an electrospray ionization (ESI) source. The capillary voltage was set to 3.0 kV and the fragmentor voltage was set to 100 V. The drying gas temperature was 350°C, the drying gas flow rate was 10 l min^{-1}, and the nebulizer pressure was 35 psi. Hydrolysis products were quantified by measuring the area under the peak in comparison to the PDA standard.

^{13}C-oleamide or AEA hydrolysis activity was determined using mouse brain membrane (50 µg) or recombinant FAAH in COS7 cells (5 µg), respectively, in PBS (100 µl). The reactions were performed in a manner similar to that described for 2-AG hydrolysis, except the incubation time was 20 min at 37°C and 300 µl 2:1 v/v $CHCl_3$:MeOH was used to quench the reaction.

DAGLα and DAGLβ hydrolysis activities were determined using recombinant V5-tagged DAGLα and DAGLβ[1] in HEK293 cells and a slightly modified procedure from that described previously[2]. Briefly, the substrate was prepared by sonicating 1-stearoyl-2-arachidonoylglycerol in HEPES buffer (50 mM HEPES with 100 mM NaCl, 5 mM CaCl2, and 0.005% v/v Triton X-100) until a turbid solution resulted. Reactions were prepared by diluting transfected HEK293 cell membranes (100 µg) into additional buffer without Triton X-100 to a volume of 75 µl. The substrate (25 µl, 500 µM final concentration) was added and the reaction was briefly sonicated for 5 sec before being incubated at 37°C for 30 min. The reactions were quenched by the addition of 400 µl 2:1 v/v $CHCl_3$:MeOH, doped with 1 nmol 1-monopentadecanoin, vortexed, then centrifuged (1,400 x *g*, 3 min) to separate the phases. LC-MS analysis of the organic phase was performed as described above, and hydrolysis products were quantified by measuring the area under the peak in comparison to the 1-monopentadecanoin standard.

$cPLA_2$ hydrolysis activity was determined using recombinant $cPLA_2$ in HEK293 cells and a slightly modified procedure from that described previously[3]. Briefly, cold PAPC, PIP_2, and ^{14}C-radiolabeled PAPC in a 96:3:1 molar ratio were mixed and dried under nitrogen in a glass vial. The substrate was prepared by diluting the dried lipids to 1 mM in HEPES buffer (100 mM HEPES pH 7.2, 0.08 mM $CaCl_2$, 0.1 mg ml^{-1} BSA, 4 mM Triton X-100) and sonicating until a clear solution resulted. Reactions were prepared by adding recombinant $cPLA_2$ (50 µg), substrate (25 µl), and additional HEPES buffer without Triton X-100 to a volume of 250 µl. After

incubating for 30 min at 37°C, the reactions were quenched by the addition of 500 µl 2:1 v/v $CHCl_3$:MeOH with 1% v/v formic acid, vortexed, and centrifuged (1,400 x *g*, 3 min) to separate the phases. The organic phase was removed, dried under a stream of nitrogen, resuspended in 20 µl $CHCl_3$, and spotted on TLC plates. The plates were developed in 2:1 Hex:EtOAc. Distribution of radioactivity on the plate was quantified by a phosphorimaging device (Packard) and the products were identified by comparison with ^{14}C-radiolabeled standards. Resulting arachidonic acid concentration was calculated from percentage values of the total radioactivity on the TLC plates.

For the MAGL and FAAH hydrolysis assays with inhibitors, the reactions were prepared as described above except without substrate. Inhibitor or DMSO was then added to the reactions in the appropriate concentrations and incubated for 30 min at 37°C. The substrate was then added and the reactions were carried out exactly as described above. For the DAGL hydrolysis assays with inhibitors, the reactions were prepared as described above except without protein. Inhibitor or DMSO was added to the proteins in the appropriate concentrations and incubated for 30 min at 37°C. The protein was then added and the reactions were carried out exactly as described above.

CB1 and CB2 receptor binding assays. Membranes were prepared from cerebellar tissue (i.e., for CB1 receptor binding) or CB2 receptor transfected CHO cells. The respective samples were placed in 5 mL cold membrane buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4) and homogenized. The samples were then centrifuged at 50,000 g at 5°C for 10 min. The supernatant was removed and samples were resuspended in 5ml buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, pH 7.4). Protein concentration was determined by Bradford method.

For CB1 receptor binding, competition curves were generated by incubating 8 mg of cerebellar membrane proteins with 3 nM [^{3}H]rimonabant in the presence or absence of JZL184 (0.001-10 µM), or WIN 55-212-2 (10 µM), which served as a positive control, for 90 min at 30°C. Non-specific binding was determined in the presence of 5 µM non radio-labeled rimonabant. The reaction was terminated by vacuum filtration though Whatman GF/B glass fiber filter that was pre-soaked in Tris buffer containing 5 g l^{-1} BSA (Tris-BSA), followed by 3 washes with 4°C Tris-BSA. Bound radioactivity was determined by liquid scintillation

spectrophotometry at 45% efficiency after extraction in ScinitSafe Econo 1 scintillation fluid. This experiment include a sample size of 6.

For CB2 receptor binding, CB2 receptor CHO cell membranes (8 mg protein), were incubated with 15 nM [^{3}H] CP-55940 in the presence or absence of JZL184 (0.0005-5 μM), or the CB2 receptor antagonist SR144528 (0.0001-1 μM), which served as a positive control in the buffer for 90 min at 30°C. The reaction was terminated by vacuum filtration though Whatman GF/B glass fiber filter that was pre-soaked in Tris buffer containing 5 g l^{-1} BSA (Tris-BSA), followed by 3 washes with 4°C Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after extraction in ScinitSafe Econo 1 scintillation fluid. A sample size of 5 was used for this experiment.

For the CB1 and CB2 receptor binding assays, data are expressed as mean and standard error for % radiolabeled ligand bound at each concentration point.

Measurement of brain lipids. One half brain was weighed and subsequently Dounce homogenized in 2:1:1 v/v/v $CHCl_3$:MeOH:Tris pH 8.0 (8 ml) containing standards for *N*-acylethanolamine (NAE), monoacylglycerol (MAG), or free fatty acid (FFA) measurements (200 pmol d_4-OEA, 20 pmol d_4-AEA, 400 pmol C15:0-MAG, and 4 nmol PDA). The mixture was vortexed and then centrifuged (1,400 x *g*, 10 min). The organic layer was removed, $CHCl_3$ was added until the final volume was again 8 ml, and the extraction was repeated. The combined organic extracts were dried under a stream of N_2 and resolubilized in 2:1 v/v $CHCl_3$:MeOH (120 μl). 30 μL and 15 μL of resolubilized lipid were injected for positive mode (MAG and NAE) and negative mode (free fatty acid) measurements, respectively. Lipid measurements were performed by LC-MS using the same instrument and solvents as described above. Solvent modifiers such as 0.1% formic acid or 0.1% ammonium hydroxide were included to assist in ion formation in positive and negative ionization mode, respectively. For positive mode measurements, the flow rate for each run started at 0.1 ml min^{-1} for 5 min. The gradient started at 0% B and increased linearly to 100% B over 40 min with a flow rate of 0.4 ml min^{-1}, followed by an isocratic gradient of 100% B for 7 min before equilibrating for 8 min at 0% B with a flow rate of 0.5 ml min^{-1}. For negative mode measurements, the flow rate for each run started at 0.1 ml min^{-1} for 3 min. The gradient started at 0% B and increased linearly to 100% B over 17 min with a flow rate of 0.4 ml min^{-1}, followed by an isocratic gradient of 100% B for 7 min before equilibrating for 3

min at 0% B with a flow rate of 0.5 ml min^{-1}. MAGs, NAEs, and FFAs were quantified by measuring the area under the peak in comparison to the C15:0 or deuterated standards. Targeted LC-MS measurements were performed using selected ion monitoring (SIM).

***In vivo* microdialysis studies.** Experimentally naïve male C57Bl/6 mice (25-27g) were each implanted with a single microdialysis probe into the nucleus accumbens that was secured to the skull with dental cement. The stereotaxic coordinates from Bregma were +1.5 mm AP; ±0.8 mm ML; -5.0 mm V (from skull) and the microdialysis probes employed a 1 mm length of active membrane (PES, 15 kDA cutoff; model 6.14.1 from SciPro Inc., Sanborn NY). Following a 12-16 h recovery period microdialysis samples were collected at 15 min intervals using a flow rate of 0.6 µl min^{-1}. Samples were collected during a 60 min baseline period, a 60 min period following injection of either JZL184 (10 mg kg^{-1}, i.p.; $n = 8$) or vehicle (4:1 v/v PEG300:Tween80, i.p.; $n = 6$), during subsequent 90 min perfusion with an aCSF containing high concentrations of KCl (150 mM) and $CaCl_2$ (10 mM) to stimulate EC release by neuronal depolarization and for 60 min during re-baseline with a standard aCSF perfusate. Details of the aCSF composition and methods for analysis of dialysate EC content by LC-MS can be found in ref. 4.

ABPP-MudPIT analysis of SH targeted by JZL184 *in vivo*. A portion of the brain membrane proteomes (1 ml, 1 mg ml^{-1} in PBS) from the mice treated with JZL184 or vehicle as described above was labeled with 5 µM FP-biotin for 1 h at room temperature and prepared for ABPP-MudPIT analysis as previously described, except that the Lys-C digestion step was omitted[5,6]. MudPIT analysis of eluted peptides was carried out as previously described on a coupled Agilent 1100 LC-ThermoFinnigan LTQ-MS instrument. All data sets were searched against the mouse IPI database using the SEQUEST search algorithm and the results were filtered and grouped with DTASELECT. Peptides with cross-correlation scores greater than 1.8 (+1), 2.5 (+2), 3.5 (+3) and delta CN scores greater than 0.08 were included in the spectral counting analysis. Only proteins for which 5 or more spectral counts were identified on average in the control samples were considered for comparative analysis. Specifically, probe-labeled proteins were further identified by their presence in FP-treated samples with a spectral number at least 5-fold or greater than that observed in "no probe" control runs (experiments performed as described above, but without

inclusion of biotinylated FP). Spectral counts are reported as the average of three samples with the standard error of the mean (SEM).

Behavioral studies. The pharmacological effects of JZL184 in wild type mice, mice treated with rimonabant, and *CB1*$^{-/-}$ mice were evaluated using the following indices: locomotor activity, nociception in the hotplate and tail immersion tests, catalepsy in the bar test, and for hypothermia. A total of four groups of mice were used, with a sample size of 14 mice per group. Before injections, the mice were evaluated for baseline responses in the tail immersion test, as well for rectal temperatures. Subjects were given an intraperitoneal injection of rimonabant or vehicle, followed by an intraperitoneal injection of JZL184 or its respective vehicle 10 min later. Locomotor activity was assessed 120 min after administration of JZL184 or vehicle, tail withdrawal latencies were evaluated 135 min after the second injection, and catalepsy and rectal temperature were assessed at 145 min post-injection. For determining hypomotility each mouse was placed in a clear Plexiglas box (42.7 x 21.0 x 20.4 cm) for a 10 min assessment period and Anymaze (Stoelting, Wood Dale, Illinois) software was used to determine the percentage of time spent immobile. Subjects were assessed for nociception in the tail immersion assay. Each mouse was placed head first into a small bag fabricated from absorbent under pads (VWR Scientific Products; 4 cm diameter, 11 cm length) with the tail out of the bag. The experimenter gently held the mouse and immersed approximately 1 cm of the tip of the tail into a water bath maintained at 56.0° C. The latency for the animal to withdraw its tail from the water within a 10 s cutoff time was scored. Catalepsy was evaluated using the bar test, in which the front paws of each subject were placed on a rod (0.75 cm diameter) that was elevated 4.5 cm above the surface. Mice that remained motionless with their paws on the bar for 10 s (with the exception of respiratory movements) were scored as cataleptic. Although no mice were found to be cataleptic in the present study, half of the mice treated with JZL184 alone displayed a hyperreflexia when the front paws were placed on rod, which was exemplified by jumping or "popcorn" behavior. Rectal temperature was determined by inserting a thermocouple probe 2.0 cm into the rectum and temperature was obtained from a telethermometer.

A second group of naïve subjects (n = 10-11 mice per group) were evaluated in the acetic acid stretching assay. Subjects were given a subcutaneous injection of rimonabant or its vehicle, followed by a subcutaneous injection of JZL184 or vehicle 10 min later. Acetic acid (0.6%) was

injected intraperitoneally in a volume of 10 µl g^{-1} body weight 120 min after the second injection. The number of stretches (constriction of abdomen, turning of trunk (twist) and extension of the body and hind limbs) per mouse was counted during a 20 min period after the administration of acetic acid.

For the formalin test, the subjects consisted of a total of 24 male C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME). Each mouse was given an intraperitoneal injection of rimonabant (3 mg kg^{-1}) or vehicle, followed by a p.o. injection of JZL184 (40 mg kg^{-1}) or its respective vehicle 10 min later. Thus, there were a total of four groups, with a sample size of 6 mice/group. Two hours after the second injection, each subject was given an intraplantar injection of 2.5% formalin solution in a volume of 20 ml into a hind paw. The total amount of time spent licking or lifting the afflicted paw was recorded for both the early phase (i.e., 0-10 min) and the late phase (i.e., 20-40 min). Peak pain behavior was observed during each respective time period in control mice. JZL184 was administered p.o. rather than i.p. to minimize the effects of vehicle on the formalin-induced behavioral responses of animals.

For studies with $CB1^{+/+}$ and $CB1^{-/-}$ mice, male mice were derived from heterozygous breeding pairs that were backcrossed onto a C57Bl/6J background for at least 13 generations. Baseline tail withdrawal latencies and rectal temperatures were obtained for all mice before drug administration. Subjects in each genotype were given i.p. injections of either vehicle or JZL184 (16 mg kg^{-1}) and 2 h later were evaluated for locomotor activity, tail withdrawal latencies, catalepsy, and rectal temperature. A total of four groups of mice were used, with a sample size of 8 mice per group.

REFERENCES

1. Bisogno, T. et al. Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J. Cell Biol.* **163**, 463-468 (2003).
2. Majerus, P.W. & Prescott, S.M. Characterization and assay of diacylglycerol lipase from human platelets. *Methods Enzymol* **86**, 11-7 (1982).
3. Yang, H.C., Mosior, M., Johnson, C.A., Chen, Y. & Dennis, E.A. Group-specific assays that distinguish between the four major types of mammalian phospholipase A2. *Anal Biochem* **269**, 278-88 (1999).
4. Caille, S., Alvarez-Jaimes, L., Polis, I., Stouffer, D.G. & Parsons, L.H. Specific Alterations of Extracellular Endocannabinoid Levels in the Nucleus Accumbens by Ethanol, Heroin, and Cocaine Self-Administration. *J. Neurosci.* **27**, 3695-3702 (2007).
5. Jessani, N. et al. A streamlined platform for high-content functional proteomics of primary human specimens. *Nat Methods* **2**, 691-7 (2005).
6. Washburn, M.P., Wolters, D. & Yates, J.R., 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotechnology* **19**, 242-7 (2001).